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(54) Title: PARB INHIBITORS

(57) Abstract: Compounds of formula 1, 2, and 3 where A_1 is $C(R_4)$or N; A_2 is $C(R_5)$ or S; R_1 is H, lower alkyl, halo, or a carbonyl; R_2 is H, lower alkyl, acyl, or forms a double bond with an adjacent ring atom; R_3 is H, lower alkyl, halo, aryl, aralkyl, acyl, lower alkenyl, or a radical or the form $(CH_2)nC(O)$-R_8, where R_8 is lower alkyl, OH, NH_2, lower alkoxy, lower aklylamino, di(lower alkyl)amino, aryl, or heterocyclyl, or forms a double bond with an adjacent ring atom; R_4 is H, lower alkyl, or forms a double bond with an adjacent ring atom, R_5 is H, lower alkyl, OH, halo, lower alkoxy, lower alkyl-thio, aryl-thio, or heterocyclyl-thio; R_6 and R_7 are each independently H, lower alkyl, OH, lower alkoxy, halo, nitro, amino, thio, acyl, lower alkylamino, acyloxy, acylamino, sulfinyl, sulfonyl, alkylsulfinyl, alkylsulfonyl, arylsulfonyl, aryl, heterocyclyl, aralkyl, or heterocyclyl-alkyl; R_{10} is H, lower alkyl, lower alkenyl, aryl, heterocyclyl, aryl-lower alkyl, or heterocyclyl-lower alkyl; and R_{11}, R_{12}, and R_{13} are each independently halo, nitro, OH, NH_2, or lower alkyl, and pharmaceutically acceptable salts thereof, are effective modulators of PARP enzymes.

PARP INHIBITORS

Field of the invention

This invention relates to the fields of molecular biology and medicinal chemistry. More particularly, the invention relates to compounds and methods for modulating the activity of PARP.

Background of the Invention

Living organisms possess mechanisms to regulate cell cycle progression and to preserve genomic integrity. Failure of these mechanisms in multicellular organisms results in disorders ranging from the unregulated cell proliferation associated with cancer, to massive cell death following the fall of tissue oxygen and glucose levels in cardiac or brain ischemia (D.W. Choi, Nat Med (1997) 3(10):1073-74) .

A key cellular response to genomic damage is the post-translational modification of nuclear proteins in response to DNA strand breaks (P.A. Jeggo, Curr Biol (1998) 8(2):R49-51; A.A. Pieper et al., Trends Pharmacol Sci (1999) 20(4):171-81). One known modification is the addition to specific proteins of up to 200 residues of ADP-ribose to form branched polymers. These polymers act as binding sites for repair proteins that play a central role in DNA metabolism (D. D'Amours et al., Biochem J (1999) 342(Pt 2):249-68). The enzyme responsible for the addition of these polymers is poly (ADP-ribose) polymerase, or PARP1. PARP1 associates with DNA and with chromatin binding proteins such as histones, transcription factors and key DNA repair proteins. Although a number of nuclear proteins such as histones are substrates for PARP1, a major substrate is PARP1 itself, via auto-modification of the BRCA1 C-terminal homology (BRCT) region. Regulation of auto-modification of PARP1 is twofold: through PARP1-DNA interactions as well as PARP1-PARP1 dimerization (H. Mendoza-Alvarez et al., J Biol Chem (1993) 268(30)22575-80).

PARP1 acts together with the DNA damage repair system to regulate DNA base excision repair, apoptosis and necrosis (D. D'Amours et al., supra). Studies of mouse strains lacking the PARP1 gene have identified two roles for this protein depending on the extent of DNA damage (V. Burkart et al., Nat Med (1999) 5(3):314-19; M. Masutani

et al., Proc Natl Acad Sci USA (1999) 96(5):2301-04; M. Masutani et al., Mutat Res (2000) 462(2-3):159-66). Moderate damage elicits a protection response similar to that observed for checkpoint genes, leaving PARP1 knockout mice vulnerable to gamma irradiation and alkylating reagents. In cases of extensive DNA damage, PARP1 activity depletes cellular energy pools, which eventually leads to cell death (V. Burkart et al., supra; A.A. Pieper, supra).

PARP1 also has a putative role in signaling DNA damage and in recruiting proteins to sites of double strand breaks. This hypothesis was based on the ability of proteins, such as p53 and other repair enzymes, to bind to the poly (ADP) polymers present on PARP1 (T. Lindahl et al., Trends Biochem Sci (1995) 20(10):405-11; D. D'Amours et al., supra). PARP1 inhibitors exaggerate the cytotoxic effects of DNA damage by limiting the ability of cells to regulate DNA base excision repair. In this role inhibitors are being tested as chemosensitizing agents during cancer chemotherapy (P. Decker et al., Clin Cancer Res (1999) 5(5):1169-72).

Another response to more extensive DNA damage mediated by PARP1 is the promotion of cell death as in cases of ischemic injury (C.a.D. Szabo, Trends Pharmacol Sci (1998) 19(7):287-98). This process can occur when PARP1 activation is highly stimulated, and thus consumes large amounts of NAD, the source of ADP-ribose. This condition depletes the cellular energy stores (K. Takahashi et al., Brain Res (1999) 829(1-2):46-54; A.A. Pieper et al., supra). PARP1 knockout mice are highly resistant to ischemia during steptozocin induced type I diabetes, myocardial infarction, stroke, and neurodegeneration (Burkart, Wang et al. 1999; Shall and de Murcia 2000) (V. Burkart et al., supra). In support of a role for PARP1 in cell death in various inflammation processes, several studies have shown protection against cellular injury in numerous target cells by using known PARP1 inhibitors (C.a.D. Szabo, supra).

For many years PARP1 has been the only known poly(ADP-ribose) polymerase. However, modification of cellular proteins with ADP-ribose polymers still occurs in PARP1 knockout mice, suggesting the presence of other proteins with PARP1 activity (M.K. Jacobson et al., Trends Biochem Sci (1999) 24(11):415-17). Indeed, new members of the PARP family have been identified based on the presence of domains that share considerable sequence similarity with the catalytic domain of PARP1 (S. Smith et

al., Science (1998) 282(5393):1484-87; J. Ame, V. Rolli et al., J Biol Chem (1999) 274(25):17860-68; M. Johansson, Genomics (1999) 57(3):442-45; V.A. Kickhoefer et al., J Cell Biol (1999) 146(5):917-28; F.R. Sallmann et al., J Biol Chem (2000) 275(20):15504-11) (see Fig. 1). PARP1 has three functional domains; a Zn^{2+}finger DNA binding sequence, an automodification domain, and a C-terminal catalytic domain. All members of the PARP1 family share a conserved catalytic domain, but a few also share a DNA binding domain or in the case of Tankyrase and VPARP, have functional domains that are not present in PARP1. Although some members of the PARP family do not possess a well-defined Zn^{2+} finger DNA binding motif or an automodification domain like that described for PARP1, they still catalyze the formation of ADP-ribose polymers in a DNA dependent manner, and are capable of automodification (Ame & Rolli et al., 1999, supra; M. Johansson, supra).

Two additional members of the PARP family are tankyrase and VPARP (S. Smith et al., 1998, supra; V.A. Kickhoefer, 1998, supra). Tankyrase is associated with the telomerase complex that is involved in aging by acting to regulate telomere length and VPARP is a component of a multisubunit complex referred to as a "Vault" (F. d'Adda di Fagagna et al., Nat Genet (1999) 23(1):76-80; L.B. Kong et al., Structure Fold Des (1999) 7(4):371-79; E. Pennisi, Science (1999) 282:1395-96). The name "Vault" is based on its observed structure by scanning electron microscopy (L.B. Kong et al., supra). The cellular location of VPARP is mostly cytoplasmic, however, there is a small fraction associated with the mitotic spindle (V.A. Kickhoefer, 1998, supra). Unlike PARP1, tankyrase and VPARP are not activated by DNA damage (S. Smith et al., 1998, supra; V.A. Kickhoefer, 1998, supra). Tankyrase modifies the telomere binding protein TRF1 *in vitro* (M.K. Jacobson et al., supra). TRF1 stabilizes the ends of chromosomes, and it has been proposed that modification of TRF1 with ADP-ribose polymers serves to regulate its ability to form a loop structure at chromosome ends (M.K. Jacobson et al., supra; E. Pennisi, supra). In other studies, tankyrase has been shown to promote telomere elongation in human cells (S. Smith et al., Curr Biol (2000) 10:1299-302). The primary substrate of VPARP is the major vault protein, MVP (it is also capable of automodification); these complexes are upregulated in multidrug resistant cancer cell lines (V.A. Kickhoefer et al., J Biol Chem (1998) 273(15):8971-74; A.B. Schroeijers et al., Cancer

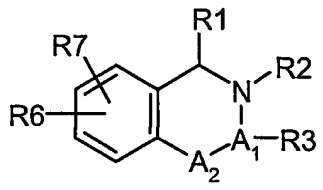
Res (2000) 60(4):1104-10). The various cellular locations and domain structures of the PARP family members strongly suggest that they have distinct cellular roles. Identification of selective inhibitors might help elucidate the function of these enzymes. Poly-(ADP-ribose) polymers can be removed by poly(ADP-ribose) glycohydrolase or PARG; a member of a large family of related enzymes (W. Lin et al., J Biol Chem (1997) 272(18):11895-901; M.K. Jacobson et al., supra; J.C. Ame and E.L. Jacobson et al., Mol Cell Biochem (1999) 193(1-2):75-81; G. Pacheco-Rodriguez et al., Mol Cell Biochem (1999) 193(1-2):13-18; T. Shimokawa et al., J Biochem (Tokyo) (1999) 126(4):748-55). This enzyme is thought to regulate the cellular function of PARP family members by removing ADP-ribose units, which results in changes in the branching pattern of the polymers (Ame & Jacobson et al., supra; M.K. Jacobson et al., supra). There is some evidence to support the hypothesis that polymers synthesized by different PARP orthologs might be hydrolyzed by specific PARGs. Although a complete understanding of the physiological activities of PARPs remains unclear, inhibitors of the activity of PARP1 and related proteins could provide new therapeutic approaches to both cancer and ischemia caused by reperfusion injury and inflammatory processes (C.a.D. Szabo, supra).

Kaiser et al. found that the constitutive expression of PARP1 in *Saccharomyces cerevisiae* is only possible with simultaneous inhibition of ADP-ribosylation activity through the addition of the known inhibitor 3-methoxybenzamide to the growth media (P. Kaiser et al., Mol Gen Genet (1992) 232(2):231-39). Induction of fully active PARP1 under the conditional galactose promoter led to growth arrest (Kaiser et al., supra; M.A. Collinge et al., Mol Gen Genet (1994) 245(6):686-93). The growth inhibition was relieved by removal of the N-terminal region of PARP1 that contains the DNA binding domain. Yeast does not possess endogenous PARP1 activity so the cause of the growth arrest is unknown. Antibodies raised against polymers of poly (ADP-ribose), were used to determine that a protein of approximately 116,000 kD, the size of PARP1 itself, was ADP ribosylated in the yeast cells expressing PARP1 (Kaiser et al., supra). This lead to the proposal that growth inhibition in yeast might occur through sequestration of chromosomal proteins by the ADP-ribose decorated PARP1 protein rather than toxicity due to direct modification of a yeast protein by PARP.

Summary of the Invention

We have now demonstrated that PARP2, a protein closely related to PARP1, also causes growth inhibition when conditionally expressed in yeast. The reduced growth phenotype was used as an assay to screen for novel inhibitors of these proteins by selecting those compounds that can restore growth to yeast expressing PARP1 or PARP2. The inhibitors identified in our screen also inhibit recombinant PARP1 activity *in vitro*. A few inhibitors show selectivity for PARP1 or PARP2. These compounds are the first that specifically recognize PARP orthologs in cells. Our data demonstrate the utility of yeast as a screening system to identify inhibitors of human genes and gene families, even when the protein is not highly conserved with any yeast proteins.

One aspect of the invention is a compound of formula 1:



where A_1 is $C(R_4)$ or N; A_2 is $C(R_5)$ or S;

R_1 is H, lower alkyl, halo, or a carbonyl;

R_2 is H, lower alkyl, acyl, or forms a double bond with an adjacent ring atom;

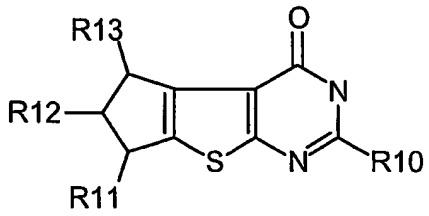
R_3 is H, lower alkyl, halo, aryl, aralkyl, acyl, lower alkenyl, or a radical of the form $-(CH_2)_nC(O)-R_a$, where R_a is lower alkyl, OH, NH_2, lower alkoxy, lower alkylamino, di(lower alkyl)amino, aryl, or heterocyclyl, or forms a double bond with an adjacent ring atom;

R_4 is H, lower alkyl, or forms a double bond with an adjacent ring atom (preferably, R_3 and R_4 do not simultaneously form double bonds with adjacent ring atoms);

R_5 is H, lower alkyl, OH, halo, lower alkoxy, lower alkyl-thio, aryl-thio, or heterocyclyl-thio;

R_6 and R_7 are each independently H, lower alkyl, OH, lower alkoxy, halo, nitro, amino, thio, acyl, lower alkylamino, acyloxy, acylamino, sulfinyl, sulfonyl, alkylsulfinyl, alkylsulfonyl, arylsulfonyl, aryl, heterocyclyl, aralkyl, or heterocyclyl-alkyl.

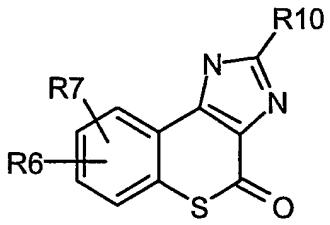
Another aspect of the invention is a compound of formula 2,



wherein R_{10} is H, lower alkyl, lower alkenyl, aryl, heterocyclyl, aryl-lower alkyl, or heterocyclyl-lower alkyl;

and R_{11}, R_{12}, and R_{13} are each independently halo, nitro, OH, NH_2, or lower alkyl, and pharmaceutically acceptable salts thereof.

Another aspect of the invention is a compound of formula 3:



wherein R_{10}, R_6 and R_7 are as defined above, and pharmaceutically acceptable salts thereof.

Another aspect of the invention is a method for inhibiting the activity of a PARP enzyme, comprising contacting said enzyme with an effective amount of a compound of formula 1, 2, or 3.

Another aspect of the invention is a composition for treating a disorder modulated by a PARP enzyme, comprising an effective amount of a compound of formula 1, 2, or 3, and a pharmaceutically acceptable carrier.

Another aspect of the invention is an assay for compounds that modulate PARP activity, comprising a eukaryotic cell that expresses a heterologous PARP and exhibits a detectable phenotype as a result of heterologous PARP expression.

Another aspect of the invention is a method for identifying compounds that modulate PARP activity, comprising providing a eukaryotic cell that expresses a heterologous PARP enzyme and exhibits a detectable phenotype as a result of said PARP expression, contacting the cell with a candidate PARP modulating compound, and determining whether the compound reverses the detectable phenotype.

Brief Description of the Figures

FIG. 1 is a diagram illustrating similarities between PARP enzymes and related proteins, by domain. The white bars represent the coding region of the genes and their length reflects the relative difference in size of the gene products. The family members each contain domains that are marked by square boxes filled in the following manner: diagonal lines (DNA binding domains), cross hatches (nuclear localization sequence), vertical lines (automodification domain) and black filled (domains not present in PARP1).

FIG. 2 is a graph depicting the results of an experiment in which S. cerevisiae expressing either wildtype PARP1 or PARP2 (large or small diagonal lines) or a catalytically inactive mutant (solid black or gray) was grown in 2% glucose (uninduced) or 2% galactose media (induced).

FIG. 3 is a graph depicting the results of an experiment in which yeast cells carrying integrated copies of PARP1 or PARP2 under control of the inducible GAL1 promoter (large and small diagonal lines, respectively) were grown in glucose (labeled repressed) and 2% galactose (labeled induced). Their growth was analyzed in the presence of episomally expressed PARG also under control of the GAL1 promoter (diagonal, solid black or horizontal lines), or a vector control (solid gray).

FIG. 4 is a graph depicting the results of an experiment in which cells expressing PARP1 or PARP2 were exposed to varying concentrations of the inhibitor 6(5H)-phenanthridinone the percentage of growth restoration is analyzed. The EC_{50} is 10.2 μM for PARP1 and 36.3 μM for PARP2 expressing cells.

FIG. 5 is a graph depicting the results of an experiment in which normal yeast strains or strains carrying a deletion of the two efflux pumps PDR5 and SNQ2, were exposed to increasing concentration of the known inhibitor 6(5H)-phenanthridinone and two analogs, ICX56242099 and ICX56209576. The wild type strain is marked with solid circles while the *pdr5*□*snq2*□ strain is marked by solid squares.

FIG. 6 is a graph of the dose response curve for two hits identified by screening which showed selectivity for either PARP1 or PARP2. The EC_{50} value for yeast expressing PARP1 by extrapolation is approximately 60 μM. The PARP1 hits were tested on

yeast expressing PARP2 and ICX56258231 showed a higher activity against PARP2 than PARP1, while ICX56259537 showed higher efficacy for PARP1.

FIG. 7 is a graph of the data obtained from an experiment in which recombinant PARP1 was assayed by determining the level of incorporation of radiolabelled NAD in a TCA-precipitable polymer composed of ADP-ribose units. All the compounds identified by screening the yeast cell-based assay inhibited the activity of PARP1 *in vitro*. An inactive analog of 6(5H)-phenanthridinone did not inhibit the activity of PARP1. Phenanthridinone (solid circles), ICX56225328 (plus signs), ICX56304405 (open rhomboids), ICX56290675 (crosses), and ICX56258231 (solid squares).

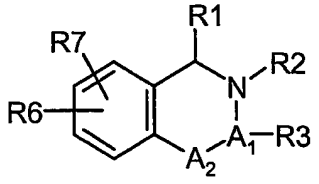
FIG. 8 is a graph of the data obtained from an experiment in which PARP1 and PARP2 activity from yeast cell extracts was assayed by determining the level of incorporation of radiolabelled NAD in a TCA-precipitable polymer composed of ADP-ribose units. No significant PARP1 or PARP2 activity was detected in glucose medium (PARP expression repressed), but when cells were grown in galactose medium (expression of PARP1 and PARP2 induced) activity was observed.

FIG. 9 depicts data evidencing that inhibitors of PARP1 and PARP2 identified in screening that inhibit PARP1 activity in vitro also inhibited the activity of PARP1 and PARP2 in yeast cell extracts. An inactive analog of 6(5H)-phenanthridinone did not inhibit the activity of either PARP1 or PARP2. Solid bars represent PARP1 activity and gray bars represent PARP2 activity. The names of the different compounds tested are displayed on the *x-axis* of the histogram.

Detailed Description

Definitions:

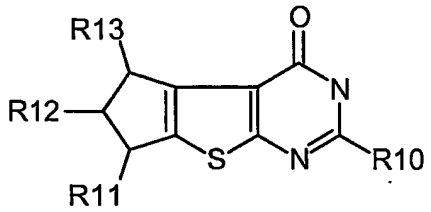
"Compound of formula 1" refers to a compound having the structure:



wherein A_1 is $C(R_4)$ or N; A_2 is $C(R_5)$ or S; R_1 is H, lower alkyl, halo, or a carbonyl; R_2 is H, lower alkyl, acyl, or forms a double bond with an adjacent ring atom;

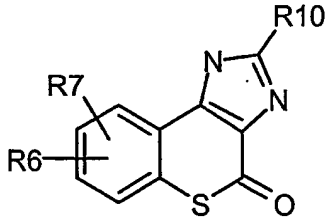
R_3 is H, lower alkyl, halo, aryl, aralkyl, acyl, lower alkenyl, or a radical of the form $-(CH_2)_nC(O)-R_a$, where R_a is lower alkyl, OH, NH_2, lower alkoxy, lower alkylamino, di(lower alkyl)amino, aryl, or heterocyclyl, or forms a double bond with an adjacent ring atom; R_4 is H, lower alkyl, or forms a double bond with an adjacent ring atom; R_5 is H, lower alkyl, OH, halo, lower alkoxy, lower alkyl-thio, aryl-thio, or heterocyclyl-thio; R_6 and R_7 are each independently H, lower alkyl, OH, lower alkoxy, halo, nitro, amino, thio, acyl, lower alkylamino, acyloxy, acylamino, sulfinyl, sulfonyl, alkylsulfinyl, alkylsulfonyl, arylsulfonyl, aryl, heterocyclyl, aralkyl, or heterocyclyl-alkyl.

"Compound of formula 2" refers to a compound having the structure:



wherein R_{10} is H, lower alkyl, lower alkenyl, aryl, heterocyclyl, aryl-lower alkyl, or heterocyclyl-lower alkyl; and R_{11}, R_{12}, and R_{13} are each independently halo, nitro, OH, NH_2, or lower alkyl, and pharmaceutically acceptable salts thereof.

"Compound of formula 3" refers to a compound having the structure:



wherein R_{10}, R_6 and R_7 are as defined above, and pharmaceutically acceptable salts thereof.

The term "lower alkyl" refers to radicals containing carbon and hydrogen, without unsaturation, having from one to six carbon atoms, inclusive. Lower alkyl radicals can be straight or branched. Exemplary lower alkyl radicals include, without limitation, methyl, ethyl, propyl, isopropyl, hexyl, t-butyl, and the like. The term "lower alkenyl" refers to a hydrocarbon radical having 2-6 carbon atoms, and at least one double bond. Exemplary lower alkenyl radicals include, without limitation, vinyl, propenyl, butenyl, and the like. The term "lower alkoxy" refers to a radical of the form RO–, where R is

lower alkyl. Similarly, "lower alkylamino" refers to a radical of the form RNH–, and "di(lower alkyl)amino" and "dialkylamino" refer to radicals of the form R_xR_yN–, where R_x and R_y are each independently lower alkyl. "Lower alkylthio" refers to a radical of the form RS–, alkylsulfinyl refers to a radical of the form RS(O)–, and alkylsulfonyl refers to a radical of the form $RS(O_2)$–.

The term "aryl" refers to an aromatic carbocyclic or heterocyclic moiety, having one, two, or three rings. Exemplary aryl radicals include, without limitation, phenyl, naphthyl, pyridyl, pyrimidyl, triazyl, quinazolinyl, pyranyl, thiazolyl, and the like. The terms "aralkyl" and "aryl-lower alkyl" refer to an aryl moiety joined to a lower alkyl moiety, for example benzyl, phenethyl, 2-phenylpropyl, naphthylmethyl, and the like.

The term "heterocyclyl" refers to a cyclic organic radical containing one or more heteroatoms, such as oxygen, nitrogen, sulfur, or phosphorus. Heterocyclyl radicals within the scope of the invention can be aromatic or non-aromatic, and can have one, two, or three rings. Suitable heterocyclyl radicals include, without limitation, furyl, tetrahydrofuryl, pyranyl, tetrahydropyranyl, pyrrolyl, imidazolyl, pyrimidyl, piperidyl, pyridyl, pyridyl N-oxide, thiofuryl, oxazolyl, thiazolyl, oxazipinyl, indolyl, benzofuranyl, benzimidazolyl, quinolyl, carbazolyl, phenothiazinyl, and the like.

The term "halo" refers to fluoro, chloro, bromo, and iodo.

The term "leaving group" refers to a radical that is easily displaced by a nucleophile in an S_N2 displacement reaction. Exemplary leaving groups include, without limitation, sulfonates such as tosylate and mesylate, silanes such as t-butyl-dimethylsilane, halogens such as bromo and chloro, and the like.

The term "pharmaceutically acceptable salts and esters" refers to derivatives of compounds of formula 1 obtained by addition of an acid or base to the compound, or condensation with an alcohol or carboxylic acid to form an ester. In either case, the acid, base, alcohol, or carboxylic acid must not be unacceptably toxic at the concentrations at which the compound is administered. Suitable acids include, without limitation, inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid or phosphoric acid; organic acids such as acetic acid, propionic acid, glycolic acid, pyruvic acid, malonic acid, succinic acid, malic acid, maleic acid, fumaric acid, tartaric acid,

citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid and the like.

The term "modulate" as used herein refers to an alteration in PARP activity, and includes both increases and decreases in activity. Modulation of activity can occur as the result of direct interaction of a compound with PARP, interaction with another compound or protein that affects PARP activity directly or indirectly, or by altering the expression of PARP or of a protein that interacts with PARP directly or indirectly.

The terms "PARP" and "PARP enzyme" refer to PARP1 and PARP2, and homologs that are related to either PARP1 or PARP2 to at least the same degree as PARP1 and PARP2 are related to each other, derived from any mammalian species.

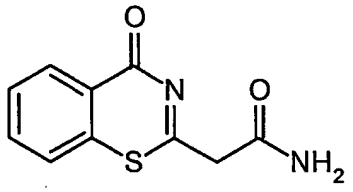
The term "active amount" refers to a quantity of compound of the invention necessary in order to create an observable change in a system. The observable change will preferably be a difference of at least 5%, more preferably at least 10%, of a measurable parameter, whether positive or negative. For example, in a screening assay that measures survival of a host cell, an active amount of compound would increase the survival rate of host cells by at least 5% over controls.

The term "effective amount" refers to the quantity of a compound of the invention necessary to produce a therapeutic effect. A therapeutic effect can be prophylaxis (prevention of symptoms from occurring in a subject at risk for developing such symptoms), treatment (amelioration or reduction of symptoms and/or the underlying cause of disease), or potentiating (increasing the susceptibility of a target cell to other agents). The precise quantity of the effective amount depends on several factors, including the condition to be treated, the condition and size of the subject, and co-therapeutic agents, and the like, but can generally be determined by one of ordinary skill in the art by routine methods.

General Method:

Compounds of the invention are prepared by standard synthetic methods. The selection of synthetic methodology depends on the particular compound selected, and is within the skill of the average practitioner in the art. In general, compounds of the invention are generated by ring-closing reactions of suitably substituted benzene derivatives.

For example, 1-((pyridine N-oxide)thio)carbonyl-2-(benzylcarbonyl)benzene is heated with hydrazine in ethanol under acid catalysis to provide 1-benzyl-4-(N-oxide pyridyl-thio)phthalazine (R_1 = benzyl, A_1 = N, A_2 = C(R_5), R_5 = N-oxide pyridylthio). Similarly, reactants such as 1-(3-amino-1,3-dioxopropylthio)-2-(chlorocarboxy)-benzene can be



cyclized under suitable conditions to yield compounds such as compound ICX56259537: Other compounds of the invention are prepared by similar methods.

Compounds of the invention can be tested for biological activity using standard methodology, such as, for example, direct biochemical assays measuring inhibition of PARP directly, cellular assays measuring inhibition of PARP activity, functional assays measuring the reversal of a phenotype caused by PARP over-expression or under-expression, and the like. A suitable assay is described in the examples below.

Compounds of the invention can be administered to a subject, or can be applied directly to cells, for example in a cell culture. If administered to a cell culture, the compound is preferably first suspended or dissolved in a suitable carrier. Suitable carriers include, without limitation, water, saline solution, dimethylsulfoxide (DMSO) and solutions thereof, cell culture media, and the like.

Useful pharmaceutical carriers for the preparation of the pharmaceutical compositions hereof can be solids or liquids. Thus, the compositions can take the form of tablets, pills, capsules, powders, sustained release formulations, solutions, suspensions, elixirs, aerosols, and the like. Carriers can be selected from the various oils, including those of petroleum, animal, vegetable or synthetic origin, for example, peanut oil, soybean oil, mineral oil, sesame oil, and the like. Water, saline, aqueous dextrose, and glycols are preferred liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, cellulose, talc, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the like. Other

suitable pharmaceutical carriers and their formulations are described in "Remington's Pharmaceutical Sciences" by E. W. Martin.

A compound of formula 1 or a pharmaceutical composition containing same is administered via any of the usual and acceptable methods known in the art, either singly or in combination with another compound or compounds of the present invention or other pharmaceutical agents. These compounds or compositions can thus be administered orally, systemically (e.g., transdermally, intranasally or by suppository) or parenterally (e.g., intramuscularly, subcutaneously and intravenously), and can be administered either in the form of solid or liquid dosages including tablets, solutions, suspensions, aerosols, and the like, as discussed in more detail above. It is preferred to administer compounds of formula 1 orally. The formulation can be administered in a single unit dosage form for continuous treatment or in a single unit dosage form ad libitum when relief of symptoms is specifically required.

PARP enzymes assist in the repair and maintenance of DNA: thus, appropriate inhibition of PARP can serve as cytotoxins, and as sensitizing agents that render target cells more susceptible to DNA-damaging therapies (such as radiotherapy and chemo-therapeutics). Disorders caused by overexpression of PARP can be treated directly with PARP inhibitors.

Examples

The following examples are provided as a guide for the practitioner of ordinary skill in the art. Nothing in the examples is intended to limit the claimed invention. Unless otherwise specified, all reagents are used in accordance with the manufacturer's recommendations, and all reactions are performed at standard temperature and pressure.

Example 1

(Materials and Methods)

(A) Yeast strains, media and methods. Isogenic derivatives of the W303 *S. cerevisiae* background were used in this study (B.J. Thomas et al., Cell (1989) 56(4):619--30; B.J. Thomas et al., Genetics (1989) 123(4):725-38) and listed below. Yeast strains lacking the major efflux pumps *PDR5* and *SNQ2* were constructed from a strain YM4 (a

gift of T. Lila, Microcide Pharmaceuticals) deleted for both via a two-step gene disruption process (T.L. Orr-Weaver et al., Meth Enzymol (1983) 101:228-45). The *pdr5*□ allele was deleted at base pair 101 through 4149 of the *PDR5* open reading frame and *snq2*□ allele was deleted for base pair 235 through 4105 of *SNQ2* resulting in strain YPB63. To construct strain EIS20-2B, YPB63 was backcrossed to W303-1α and retention of the *pdr5*□ *snq2*□ alleles was confirmed by whole cell PCR and increased sensitivity to cyclohexamide; a toxic pump substrate. YAN100 is an EIS20-2B derivative that contains a complete deletion of the *HIS3* gene. The yeast media protocols and genetic molecular biology techniques used for these studies are standard protocols (F. Sherman, Meth Enzymol (1991) 194:3-21).

The genotype of the strains is as follows: EIS20-2B and YPB63*: MATa ade2-1 his3-1115 leu2-3,112 trp1-1, ura3-1, can1-100, pdr5*□*, snq2*□; YAN100: *MATa ade2-1 his3ö::KanMx, leu2-3,112 trp1-1, ura3-1, can*^{r}*1-100, pdr5*□*, snq2*□; W303α (= W303-1B*): MATα ade2-1 his3-11,15 leu2-3,112 trp1-1, ura3-1, can1-100, pdr5*□*, snq2*□; YM4: *MATa ade2-101 his3ö200 leu2-3,112 trp1-1 ura3-52.*

(B) Cloning and analysis of PARP1, PARP2 and PARG. The cDNAs corresponding to the complete open reading frames of PARP1 and PARP2 were PCR amplified from pooled total cDNAs initially synthesized from placental, fetal brain and fetal liver poly(A)$^+$ mRNAs (Clontech Laboratories, Inc.). First and second strand cDNA synthesis was performed using Superscript II reverse transcriptase (Life Technologies, Inc.) as previously described (E.L. Perkins et al., Proc Natl Acad Sci USA (1999) 96(5):2204-09). The 5' and 3' oligonucleotides for PARP1 PCR amplification were, respectively, YS5PRP (5'GTTAATATACCTCTATACTTTAACGTCAAGGAGAAAAAACGGGAGGATGG-CGGAGTCTTCGGATAAG) and YS3PRP (5'TGAATGTAAGCGTGACATAACTAATTACA-TGATGCGGCCCTCCTCTCCCAATTACCACAGGGAGGTC) and for PARP2 were 5YSADP2lac (5' GTTAATATACCTCTATACTTTAACGTCAAGGAGAAAAAACGGAAT-TGTGAGCGGATAACAATGGCTCCAAAGCCGAAGCCCTGGGTAC) and 3YSADP2 (5' T-GAATGTAAGCGTGACATAACTAATTACATGATGCGGCCCTCGGGCACTCAGAGGTGGAC-CTCCAGC). The oligonucleotide 5YSADP2lac contains the lacO operator site. PCR amplification was carried out using either Bio-X-ACT (Bioline USA Inc., Kenilworth, NJ) or pfuTurbo (Stratagene Inc., La Jolla, CA) high fidelity thermostable DNA

polymerases according to the manufacturer instructions. All oligonucleotides were designed to amplify their target cDNAs and carry approximately 40 base pairs of homology at their 5' ends with the yeast expression vector pYES2. The pYES2 vector (Invitrogen Inc., Carlsbad, CA) contains the yeast GAL1 promoter, CYC1 transcription terminator, 2μ replication origin and the URA3 gene.

After PCR, the amplified target cDNAs were agarose gel purified. Approximately 50 ng of target cDNA was cotransformed with approximately 200 ng of *Eco*RI digested pYES2 into W303-1α and transformants were selected for uracil prototrophy. Homologous recombination of the target cDNAs into pYES2 was confirmed by yeast whole cell PCR. After the synthetic lethal phenotype of the expressed PARP1 and PARP2 was verified, plasmids from at least three independent transformants were subsequently rescued into the E. coli strain, XL10-GOLD (Stratagene Inc., La Jolla, CA), via electroporation, and further characterized by restriction enzyme analysis and DNA sequencing of the 5' and 3' cloning junctions. These studies verified the presence of the desired structures.

During the course of this work it was noted that the pYES2-PARP2 plasmid in E. coli was toxic (data not shown), thus the primer 5YSADP2lac incorporated a lacO repressor binding site; this established the plasmid during its propagation in XL10-GOLD (which carries the $lacI^q$ gene). This vector construction still exhibited instability during growth in yeast. Thus the GAL1-lac-PARP2-CYC1 terminator cassette was recombinationally cloned into the yeast URA3 2μ vector YEP24 (D. Botstein et al., Gene (1979) 8(1):17-24) using the oligonucleotides Y24smacyct (5' TCACAAATTAGAGCTTCAATTTAATTATATCAGTTATTACCCGGGGGCGCGCCGCAAATTAAAGCCTTCGAGC) and Y24pvu2gal1 (5' GGGCGAGCCGCCGAAGATTAGGCAAATTTGGTCGACGGAGCGCGCAAAGCCACTACTGCCACTTTTGGAGACTGT) effectively putting the cassettes between the *Sma*I and *Pvu*II sites of YEP24, and eliminating the tetracycline resistance gene.

For compound screening and subsequent genetic testing, the PARP1 and PARP2 expression cassettes were integrated into the indicated strains using the pARC series of dual episomal/integrative plasmid constructs. A brief summary will be presented in lieu of a detailed description of the steps required to construct the pARC series of vectors. The vectors are derived from the pRS series of vectors (R.S. Sikorski et al., Genetics

(1989) 122(1):19-27; T.W. Christianson et al., Gene (1992) 110(1):119-22). The basic elements of the pARC plasmids include a 713 base pair *Sph*I/*Bam*HI fragment containing the *S. cerevisiae GAL1* promoter and a 243 base pair *Bgl*II/*Hind*III fragment containing the *GAL4* terminator region. This region surrounds a 45 base pair polylinker that contains unique sites for *Pst*I, *Sal*I, *Spe*I, *Xho*I and *Ava*I and replaces the pRS polylinker: the base vector and polylinker of the pRS vectors is pBLUESCRIPT II. Both CEN and 2μ based vectors were generated with 44 base pairs of the 5' end of the *LYS2* gene (base pairs 8 –52 of the *LYS2* open reading frame) and 43 base pairs of the 3' end of the *LYS2* gene (base pairs 4133-4176 of the *LYS2* ORF) flanking the CEN-ARSH4 element or the 2μ replication origin. In addition, *Sfi*I sites flank the CEN-ARSH4 or 2μ elements such that digesting with this restriction enzyme liberates the elements and allows the subsequent integration into the endogenous *LYS2* locus via a "gamma" deletion mechanism (R.S. Sikorski et al., supra). For this work, integrants were selected for alpha-aminoadipate resistance and by marker prototrophy, *e.g.* Leu+ or Ura+, integration was confirmed by PCR.

For integration of PARP1, a *Spe*I/*Mlu*I (the *Mlu*I site made blunt with Klenow) GAL1-PARP1-CYC1 terminator fragment from pYES2-PARP1 was ligated into a*Spe*I/*Eco*RI cut pARC35A, a CEN-ARSH4 *LEU2* vector containing the original pBluescript II polylinker, these steps resulted in plasmid pARC35APARP1. Digestion with *Sfi*I allowed integration of the PARP1 expression cassette into the *LYS2* locus. For integration of PARP2, the GAL1-PARP2-CYC1 terminator fragment from pYES2-PARP2 was amplified by PCR using Pfu Turbo with primers YSPRC (5' GATGTATAAATGAAAGAAATTGAGATGGTGCACGATGCACAGTTGTGAATGTAAGCGTGACATAACTAATTAC; a primer containing homology at the 5' end to the GAL4 terminator) and GLUAS1 (5' TGAAGTACGGATTAGAAGCCGCCG; a primer with homology to the GAL1 UAS element). This fragment was co-transformed into yeast with *Xho*I digested pARC25B (2μ *LEU2 GAL1* promoter and *GAL4* terminator). As before, the recombinants were verified by phenotypic analysis and PCR prior to rescue into E. coli. The resulting pARC25BPARP2 plasmid was digested with *Sfi*I and integrated into the *LYS2* locus.

Phenotypic expression of the PARP1 and PARP2 clones was confirmed by constructing GFP fusions to the carboxy-terminal end of the proteins using GFP-kanMX cassettes (A.A. Wach et al., Yeast (1994) 10(13):1793-808; A.A. Wach et al., Yeast (1997) 13(11):1065-75; M.S. Longtine et al., Yeast (1998) 14(10):953-61). Expression via fluorescence microscopy and Western Blot analysis was assessed. All active site mutations were confirmed by sequencing.

PARG was isolated and cloned using primers 5YSPARG (5' GTTAATATACCT-CTATACTTTAACGTCAAGGAGAAAAAACATGAATGCGGGCCCCGGCTGTGAACC) and 3YSPARG (5' TGAATGTAAGCGTGACATAACTAATTACATGATGCGGCCCTCTCAGG-TCCCTGTCCTTTGCCCTGAATGGTC) were used to amplify the complete open reading frame from a testes cDNA library (E.L. Perkins et al., supra). The purified PCR product was co-transformed with digested pYES2 plasmid into EIS20-2B and the resulting recombinants were confirmed using whole cell PCR. Transformed isolates were subsequently reisolated and transformed into *E. coli* XL10-GOLD and confirmed by restriction enzyme analysis resulting in plasmid pYES2-PARG. To assess whether expressed PARG could suppress the synthetic lethality caused by PARP1 and PARP2, plasmid pYES2-PARG was transformed into EIS20-2B cells containing an integrated chromosomal copy of PARP1 or PARP2. Purified transformants were subsequently inoculated into galactose containing media and their growth assayed.

(C) PARP Activity Assays. A PARP Activity Assay Kit (Trevigen, Inc., Gaithersburg, MD) was used to measure the functional activity of human PARP1 and PARP2. Human PARP1 supplied in the Activity Assay Kit from Trevigen, Inc., or crude cell extracts of yeast expressing a cDNA encoding the full-length human PARP1 or PARP2 gene were used in the assay. The activity is measured by determining the level of incorporation of radiolabelled NAD in a TCA precipitable polymer composed of ADP-ribose units.

Crude cell lysates were prepared as described by M.A. Collinge et al., supra. Protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Inc., Hercules, CA). Quantitative values for PARP activity were determined by scintillation counting of the acid-insoluble counts using 20 μg total protein from crude cell lysates in a reaction containing 1 mM NAD, 1 mg/ml histones, 2 mCi ^{32}P-NAD, and 10 μg sheared

salmon testes DNA. The timed enzymatic reaction was initiated by the addition of the cell lysates, incubated at room temperature, and stopped by the addition of 20% TCA to precipitate ribosylated proteins. The protein precipitate was suspended in liquid scintillation fluid and analyzed using a scintillation counter. Each reaction was done in triplicate and the results expressed as the mean counts per minute.

(D) Compound screening of PARP1 and PARP2 inhibitors. Strain YPB63 containing integrated PARP1 was screened against a pilot library of 16,000 small molecular weight organic compounds as well as 22 compounds chosen based on computed 3D structural similarity to the known inhibitor 6(5H)-phenanthridinone. The 22 computationally selected compounds were chosen from the Iconix corporate library based on morphological similarity. The algorithms defining this methodology have been previously described and they form the basis of a rapid chemical structural similarity search method (A.N. Jain, J Comput Aided Mol Des (2000) 14(2):199-213). The pilot library represents a small but diverse collection derived from multiple commercial sources. All screening was done in duplicate at initial compound concentrations of 10 μg/ml. Cells were grown overnight in synthetic media with 2% glucose (repressed) to late logarithmic/early stationary phase. The next day cells were washed once with synthetic media without a carbon source and diluted to a final OD_{600} of 0.04 in synthetic media containing 2% galactose (induced). The diluted cells (90 μl) were added immediately to 96-well plates containing the test compound. The final volume in each well was 100 μl, and contained DMSO at a final concentration of 1%. As a control, cells containing vector (YPB63 with integrated vector plasmid) were similarly grown, washed and diluted to the same OD and then inoculated.

The plates were incubated at 30°C for 40-42 hrs, and the OD_{600} was read with a microtiter plate reader (Molecular Device, Menlo Park, CA) after shaking. The effect of compounds was measured as percent of growth restoration using the following equation: Percent Growth Restoration = (TEST - MEDarc)/(MEDvec - MEDarc) × 100, where TEST is the OD_{600} of the well with test compound, MEDarc is the median value of OD_{600} of the cells without compound, and MEDvec is the median value of OD_{600} of vector-containing cells. Compounds showing □10% of growth restoration in duplicate tests were scored as hits.

(E) Hit confirmation: Hit compounds identified from the primary screening were confirmed by generating a dose response curve using YAN100 cells expressing either PARP1 or PARP2. For the confirmation test the compounds were solubilized from powder and serially diluted (usually from 128 μM to 0.125 μM). The compounds were tested against a vector control strain (YAN100 carrying the integrated vector) and other isogenic strains expressing different cDNAs that also elicit synthetic lethal phenotypes but which are not related to PARP1.

Example 2

(Results)

(A) Growth inhibition of yeast cells expressing the human genes PARP1 or PARP2 is relieved by mutations in the active site. As shown in Fig. 2, yeast harboring an expression vector carrying the cDNA for wild-type PARP1 or PARP2 grew in 2% glucose (uninduced), but PARP1 or PARP2 expression caused growth inhibition when the cells were grown in 2% galactose (induced). To ascertain whether the inhibitory effect of PARP1 and PARP2 expression on yeast cell growth depends on their catalytic activity, a single mutation was generated in the conserved active site of the two enzymes. For PARP1, a glutamic acid was changed to an alanine at residue 988 (E988A) and the analogous change was engineered at residue 509 (E509A) in PARP2. Yeast expressing the mutant constructs grew equally well in 2% glucose or 2% galactose. Thus, the growth inhibition observed in yeast requires the catalytically active form of PARP1 or PARP2. Fluorescence microscopy revealed that expressed mammalian PARP1 and PARP2 accumulated in the nucleus.

Yeast cell growth was severely inhibited whether PARP1 and PARP2 were expressed episomally, or from a chromosomal locus (see Fig. 2 and Fig. 3). In our initial characterization of the growth phenotype caused by PARP1 or PARP2, the proteins were episomally expressed, whereas yeast carrying a chromosomal copy of PARP1 or PARP2 were used for compound screening.

(B) Expression of the human Poly (ADP-ribose) glycohydrolase (PARG) in yeast reverses the growth inhibition caused by PARP1 or PARP2 expression. To determine whether PARG reverses the growth defect caused by human PARP1 or PARP2

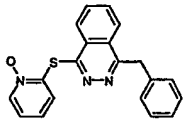
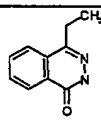
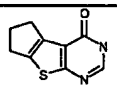
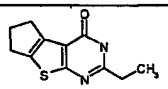
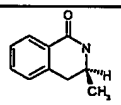
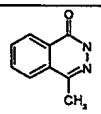
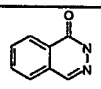
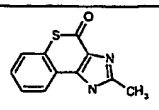
expressed in yeast, we conditionally co-expressed PARG from a high copy plasmid in yeast cells carrying an integrated copy of PARP1 or PARP2 also under control of the inducible galactose promoter. Yeast cells were grown in glucose medium (uninduced) and then switched to 2% galactose (induced) for 40 hours in synthetic media lacking uracil. As a control, yeast carrying an integrated copy of PARP1 or PARP2 and harboring a high copy plasmid with no cDNA was also tested for growth in glucose and galactose media. Fig. 3 shows the growth of yeast in the presence and absence of PARG expression. Yeast expressing PARP1 or PARP2 alone only grew at approximately 1% of the normal wild type growth levels. However, when PARG was expressed in the presence of PARP1 and PARP2 expression, growth was restored to approximately 70% of wild-type levels. As shown in Fig. 3, PARG expression alone had little effect on the growth rate of yeast (compare growth in yeast with no integrated cDNA grown in glucose and Gal medium). It is likely that PARG expression reverses the deleterious effects of PARP expression by catabolizing the ADP-ribose polymers created by PARP. The inability of PARG expression to completely counteract the effect of PARP1 expression may be due to the residual ADP-ribose monomer that is removed in mammalian cells by an ADP-ribosyl protein lyase (D.S. D'Amours et al., supra).

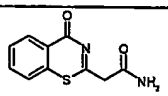
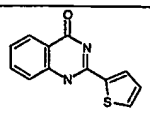
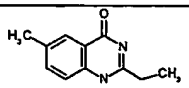
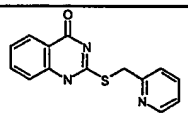
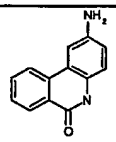
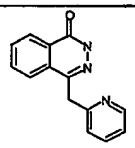
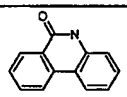
(C) A known inhibitor of PARP1, phenanthridinone, reversed the growth inhibition caused by the expression of PARP1 and PARP2. To screen for small molecule inhibitors of PARP1 and PARP2, yeast strains expressing these genes from a chromosomal locus were employed. The chromosomal expression of the genes increases the robustness of the screen by not allowing variation of plasmid copy number.

To further validate that the phenotype of growth inhibition observed in the integrated strains was due to the activity of PARP1 and PARP2, and that this phenotype would be amenable to compound screening (i.e., reversed by compounds) we determined whether the known chemical inhibitor 6(5H)-phenanthridinone would reverse the growth inhibition (Fig. 4). Yeast carrying a chromosomal copy of PARP1, PARP2 or no human cDNA were exposed to varying concentrations of 6(5H)-phenanthridinone (0 μM to 128 μM, in triplicate). Cell growth was restored when yeast were exposed to increasing concentrations of 6(5H)-phenanthridinone: the EC_{50} value for PARP1 was 10.2 μM and 36.3 μM for PARP2.

(D) Analogs of 6(5H)-phenanthridinone do not discriminate between PARP1 and PARP2. The ability of computationally chosen analogs of 6(5H)-phenanthridinone to reverse the growth inhibition caused by PARP1 and PARP2 expression was measured. Six of the twenty-two chosen analogs were active and restored growth of yeast strains expressing PARP1 (three are shown in Table 1: ICX56316703, ICX56259835, and ICX56274004). Not surprisingly, as in the case of the known PARP1 inhibitor compound, 6(5H)-phenanthridinone, the three active analogs that were tested on PARP1 and PARP2 restored the growth of yeast to a similar degree.

TABLE 1

Compound No.	Structure	PARP1 EC_{50} (μM)	PARP2 EC_{50} (μM)
ICX56258231		59.1	17
ICX56316703		39.6	37.7
ICX56290675		5.5	41
ICX56244215		70.6	70
ICX56259835		80.8	72.4
ICX56274004		41.5	77.3
ICX56225770		~100	113
ICX56222404		23.7	>500

Compound No.	Structure	PARP1 EC_{50} (μM)	PARP2 EC_{50} (μM)
ICX56259537		40	>500
ICX56304405		20.4	>500
ICX56280834		>500	>500
ICX56293720		21.5	>500
ICX56209576		6	ND
ICX56242099		~200	ND
Phenanthridinone		10.2	36.3

(E) Sensitivity of Yeast to analogs of 6(5H)-phenanthridinone is increased in strains lacking the two major efflux pumps, PDR5 and SNQ2. To optimize the sensitivity of the yeast strain to the screening compounds, mutations were made in the two efflux pumps, PDR5 and SNQ2 (M.A. Kolaczkowski et al., Microb Drug Resist (1998) 4(3):143-58). Pdr5p and Snq2p are the major efflux pumps in S. cerevisiae that confer resistance to several unrelated fungal growth inhibitors. To examine whether yeast strains lacking these two major transporters were more sensitive to the active analogs of 6(5H)-phenanthridinone, the activity of the analogs were evaluated in *pdr5*□ and *snq2*□ strains (YPB63; lacking the two pumps) or (W303; wild type for PDR5 and SNQ2) (see Fig. 5).

While four analogs showed a similar effect regardless of the presence or absence of the two efflux pumps, yeast lacking the efflux pumps were more sensitive to 6(5H)-phenanthridinone, ICX56209576 and ICX56242099 than wild-type yeast (Fig. 5). The

results underscore that the use of yeast strains lacking efflux pumps for screening compounds is beneficial in some cases.

(F) Selectivity is observed among the compounds identified as "hits" in the yeast cell-based screen. To identify new classes of inhibitors, we screened the yeast strain carrying integrated PARP1 against a 16,000 member pilot library of small organic "drug like" compounds for their ability to restore growth while PARP1 was expressed (in the presence of galactose). Ten hits showed dose dependent growth restoration on cells expressing PARP1 (see Fig. 6 and Table 1: the computationally chosen analog ICX56259835 was also identified as a screening hit). We then tested the ability of the confirmed hits to restore growth to yeast expressing PARP2. Unlike the analogs of 6(5H)-phenanthridinone, some of the ten compounds identified by screening showed patterns of selectivity for PARP1 or PARP2 (Table 1).

One inhibitor class showed modest potency, ICX56225770, ICX56244215, ICX56280834, ICX56290675, whereas one compound in this group, ICX56290675, showed some selectivity for PARP1. ICX56222404 and ICX56259537 are novel inhibitors with more pronounced selectivity for PARP1. Finally, ICX56258231 showed selectivity for PARP2 (Fig. 6). Although a clear difference in the rate of growth restoration was observed for PARP1 versus PARP2 in the presence of ICX56258231, an accurate EC_{50} value for PARP1 could not be determined. Normally this value is derived from a curve fit to a 4-parameter logistic equation, but in this case at the highest concentration of soluble compound, a plateau in the growth restoration curve was not reached.

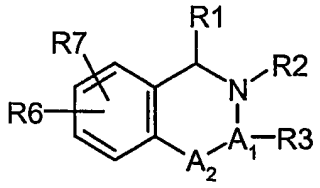
(G) Compounds that restored growth in the yeast cell-based assay inhibit purified PARP1 *in vitro*. To clarify whether the small molecules identified in screening directly inhibited PARP1 activity, the hits were tested for their ability to inhibit human recombinant PARP1 in a biochemical assay. This assay measures the incorporation of radiolabelled ribose derived from NAD into PARP1. Baseline activity was established by measuring incorporation from the radiolabelled ribose in the absence of the inhibitors.

Dose response curves were constructed for three of the compound hits (ICX56304405, IC290675, ICX56258231 and included 6(5H)-phenanthridinone and its inactive analog, ICX56 225328; Fig. 7). These results show clearly that the compounds identified in the yeast screen are direct inhibitors of PARP1.

(H) Compounds that restored growth activity also inhibit the activity of PARP1 and PARP2 from yeast cell extracts. The activity of PARP1 or PARP2 in extracts from cells grown to log phase over 8 hours in 2% glucose (uninduced) was negligible. However, cells grown under similar conditions but with 2% galactose (induced) showed an increase of 20× for PARP1 and 10× for PARP2 activity (Fig. 8). The screening compounds were then tested for their ability to inhibit the activity of PARP1 and PARP2 observed in the yeast extracts. All the compounds inhibited PARP activity as effectively as the known inhibitor with the exception of the inactive analog, ICX56225328 (Fig. 9).

What is claimed:

1. A compound of formula 1:



where A_1 is $C(R_4)$ or N; A_2 is $N(R_5)$, $C(R_5)$ or S;

R_1 is H, lower alkyl, halo, or a carbonyl;

R_2 is H, lower alkyl, acyl, or forms a double bond with an adjacent ring atom;

R_3 is H, lower alkyl, halo, aryl, aralkyl, acyl, lower alkenyl, or a radical of the form $-(CH_2)_nC(O)-R_a$, where R_a is lower alkyl, OH, NH_2, lower alkoxy, lower alkyl-amino, di(lower alkyl)amino, aryl, or heterocyclyl, and n is a whole number from 0 to 6 inclusive, or R_3 forms a double bond with an adjacent ring atom;

R_4 is H, lower alkyl, or forms a double bond with an adjacent ring atom;

R_5 is H, lower alkyl, OH, halo, lower alkoxy, lower alkyl-thio, aryl-thio, or heterocyclyl-thio;

R_6 and R_7 are each independently H, lower alkyl, OH, lower alkoxy, halo, nitro, amino, thio, acyl, lower alkylamino, acyloxy, acylamino, sulfinyl, sulfonyl, alkylsulfinyl, alkylsulfonyl, arylsulfonyl, aryl, heterocyclyl, aralkyl, or heterocyclyl-alkyl;

and pharmaceutically acceptable salts thereof.

2. The compound of claim 1, wherein R_6 and R_7 are each H.

3. The compound of claim 2, wherein A_1 is N, R_2 and R_4 each form a double bond with an adjacent ring atom, and A_2 is $C(R_5)$.

4. The compound of claim 3, wherein R_1 is carbonyl, and R_5 is H, lower alkyl, or aralkyl.

5. The compound of claim 3, wherein R_1 is benzyl, and R_5 is (N-oxide)-pyridyl-thio.

6. The compound of claim 2, wherein A_1 is $C(R_4)$.

7. The compound of claim 6, wherein A_2 is S and R_4 is a radical of the form $-(CH_2)_nC(O)-R_a$.

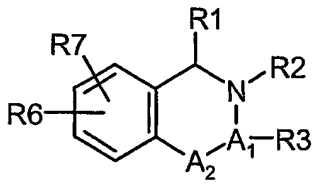
8. The compound of claim 6, wherein A_2 is $N(R_5)$.

9. The compound of claim 8, wherein R_5 is H and R_4 is 2-thiophene.

10. The compound of claim 8, wherein R_5 is H and R_4 is benzyl-thio.

11. A method for inhibiting the activity of a PARP enzyme, comprising:

contacting a PARP enzyme with an effective amount of a compound of formula 1:



where A_1 is $C(R_4)$ or N; A_2 is $N(R_5)$, $C(R_5)$ or S;

R_1 is H, lower alkyl, halo, or a carbonyl;

R_2 is H, lower alkyl, acyl, or forms a double bond with an adjacent ring atom;

R_3 is H, lower alkyl, halo, aryl, aralkyl, acyl, lower alkenyl, or a radical of the form $-(CH_2)_nC(O)-R_a$, where R_a is lower alkyl, OH, NH_2, lower alkoxy, lower alkyl-amino, di(lower alkyl)amino, aryl, or heterocyclyl, and n is a whole number from 0 to 6 inclusive, or R_3 forms a double bond with an adjacent ring atom;

R_4 is H, lower alkyl, or forms a double bond with an adjacent ring atom;

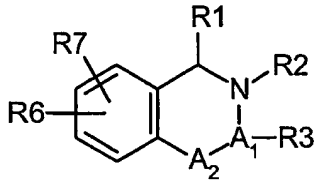
R_5 is H, lower alkyl, OH, halo, lower alkoxy, lower alkyl-thio, aryl-thio, or heterocyclyl-thio;

R_6 and R_7 are each independently H, lower alkyl, OH, lower alkoxy, halo, nitro, amino, thio, acyl, lower alkylamino, acyloxy, acylamino, sulfinyl, sulfonyl, alkylsulfinyl, alkylsulfonyl, arylsulfonyl, aryl, heterocyclyl, aralkyl, or heterocyclyl-alkyl;

or pharmaceutically acceptable salts thereof.

12. A method for inhibiting the activity of PARP2, comprising:

contacting PARP2 with an effective amount of a compound of formula 1:



where A_1 is $C(R_4)$ or N; A_2 is $N(R_5)$, $C(R_5)$ or S;

R_1 is H, lower alkyl, halo, or a carbonyl;

R_2 is H, lower alkyl, acyl, or forms a double bond with an adjacent ring atom;

R_3 is H, lower alkyl, halo, aryl, aralkyl, acyl, lower alkenyl, or a radical of the form $-(CH_2)_nC(O)-R_a$, where R_a is lower alkyl, OH, NH_2, lower alkoxy, lower alkyl-amino, di(lower alkyl)amino, aryl, or heterocyclyl, and n is a whole number from 0 to 6 inclusive, or R_3 forms a double bond with an adjacent ring atom;

R_4 is H, lower alkyl, or forms a double bond with an adjacent ring atom;

R_5 is H, lower alkyl, OH, halo, lower alkoxy, lower alkyl-thio, aryl-thio, or heterocyclyl-thio;

R_6 and R_7 are each independently H, lower alkyl, OH, lower alkoxy, halo, nitro, amino, thio, acyl, lower alkylamino, acyloxy, acylamino, sulfinyl, sulfonyl, alkylsulfinyl, alkylsulfonyl, arylsulfonyl, aryl, heterocyclyl, aralkyl, or heterocyclyl-alkyl;

or pharmaceutically acceptable salts thereof.

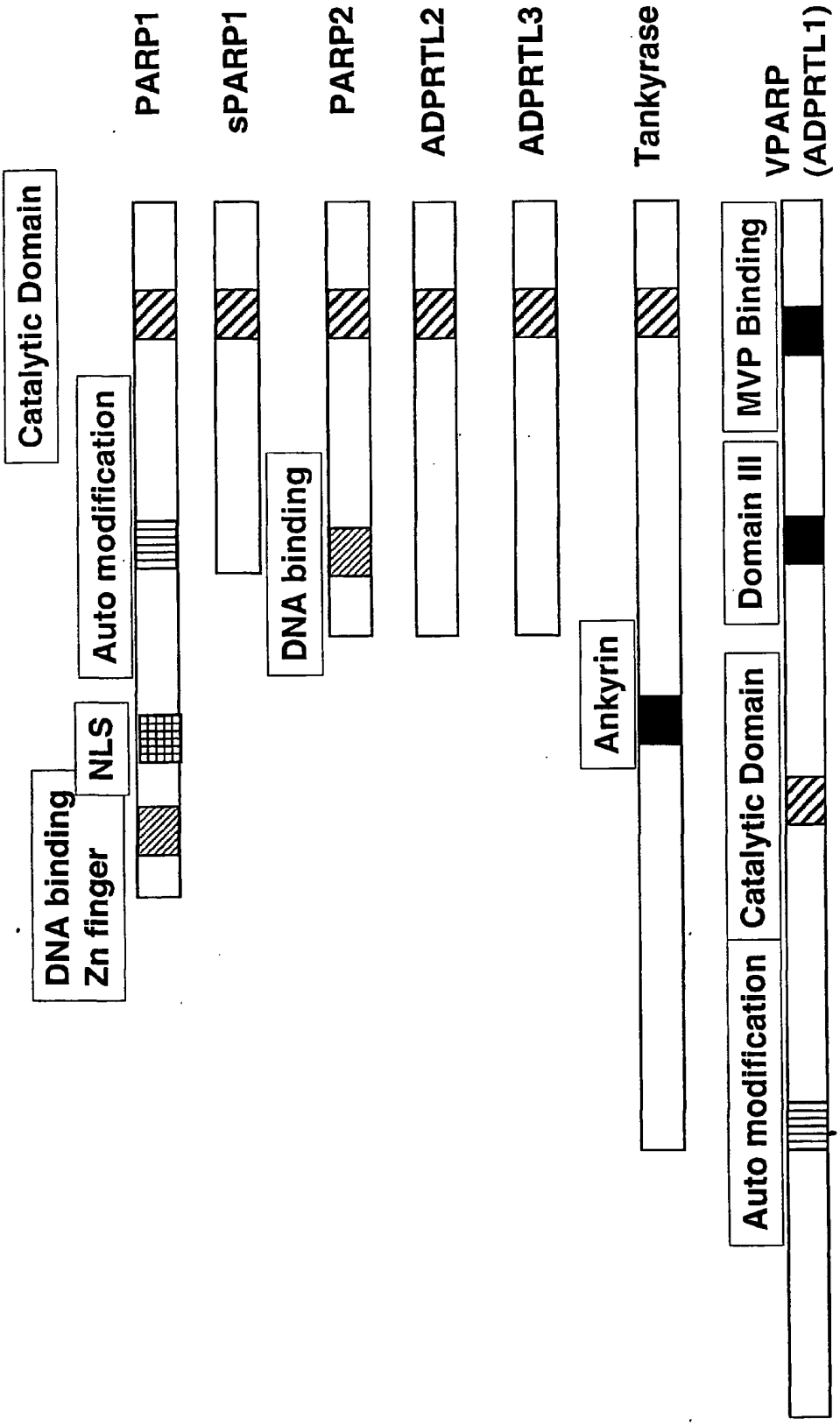

PARP1
sPARP1
PARP2
ADPRTL2
ADPRTL3
Tankyrase
VPARP (ADPRTL1)
Catalytic Domain
Auto modification
DNA binding Zn finger
NLS
DNA binding
Ankyrin
Domain III
MVP Binding
Auto modification
Catalytic Domain

Fig. 1

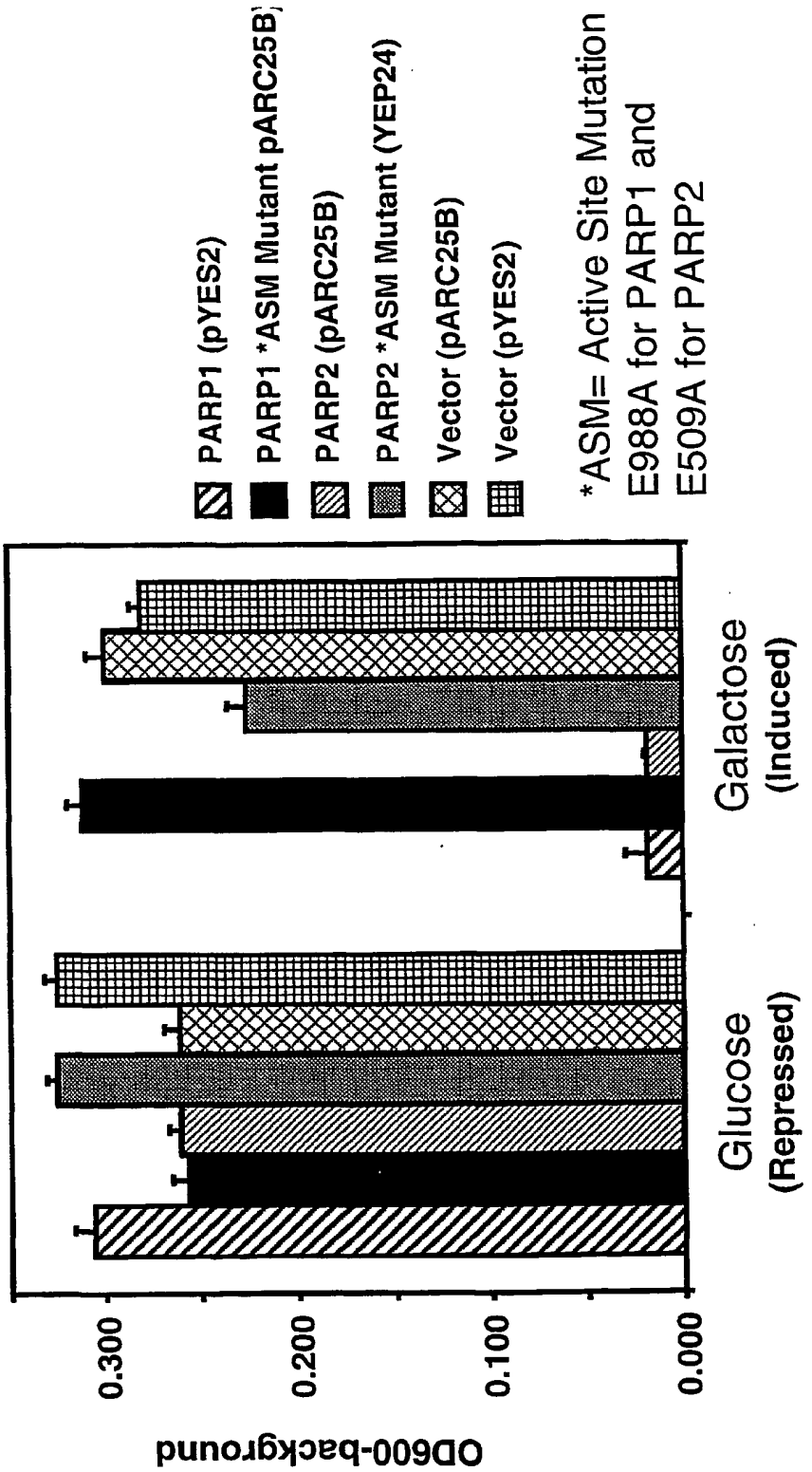

PARP1 (pYES2)
PARP1 *ASM Mutant pARC25B
PARP2 (pARC25B)
PARP2 *ASM Mutant (YEP24)
Vector (pARC25B)
Vector (pYES2)
*ASM= Active Site Mutation
E988A for PARP1 and
E509A for PARP2
Galactose
(Induced)
Glucose
(Repressed)
0.300
0.200
0.100
0.000
OD600-background

Fig. 2

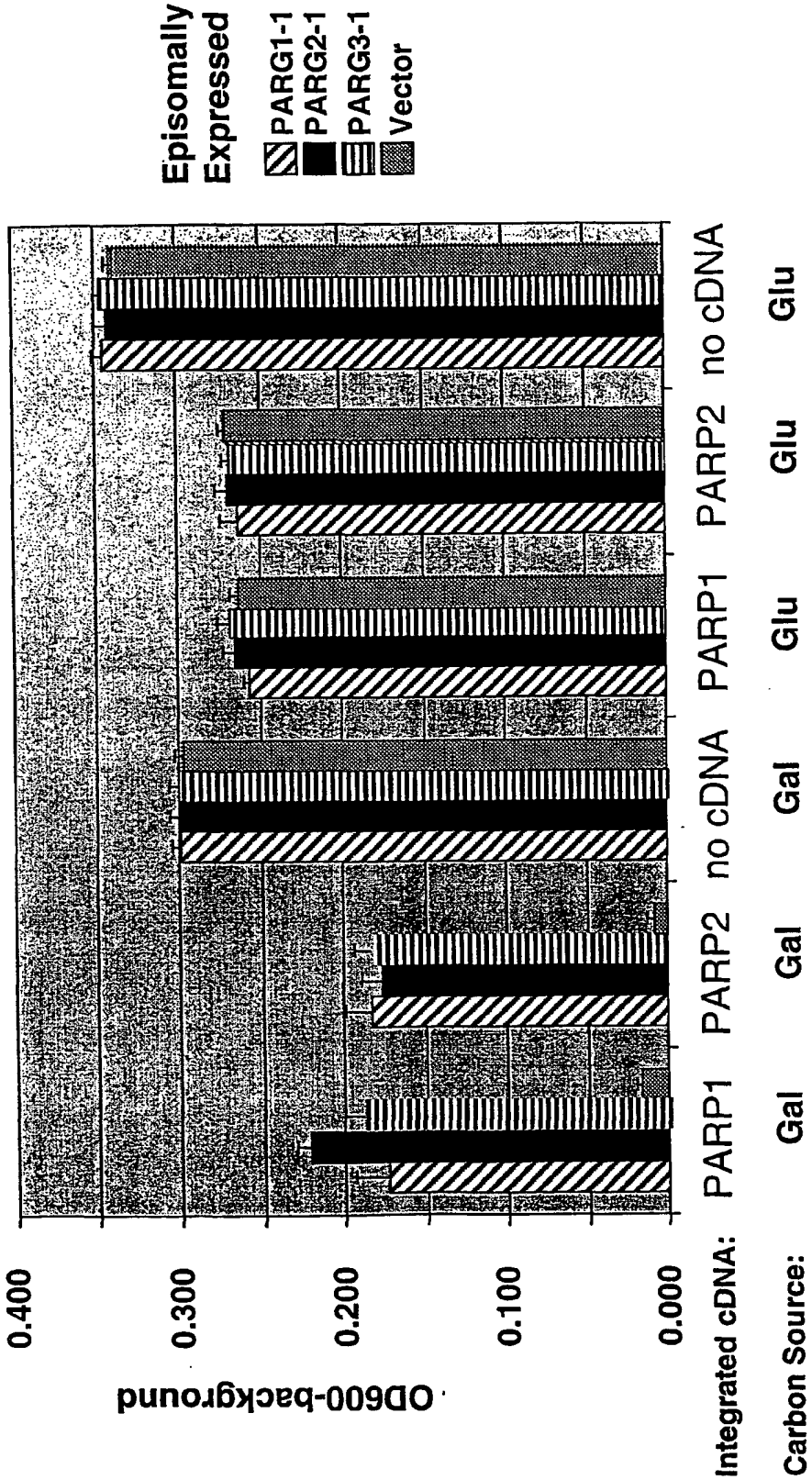

Episomally Expressed
PARG1-1
PARG2-1
PARG3-1
Vector
OD600-background
0.400
0.300
0.200
0.100
0.000
Integrated cDNA: PARP1 PARP2 no cDNA PARP1 PARP2 no cDNA
Carbon Source: Gal Gal Gal Glu Glu Glu

Fig. 3

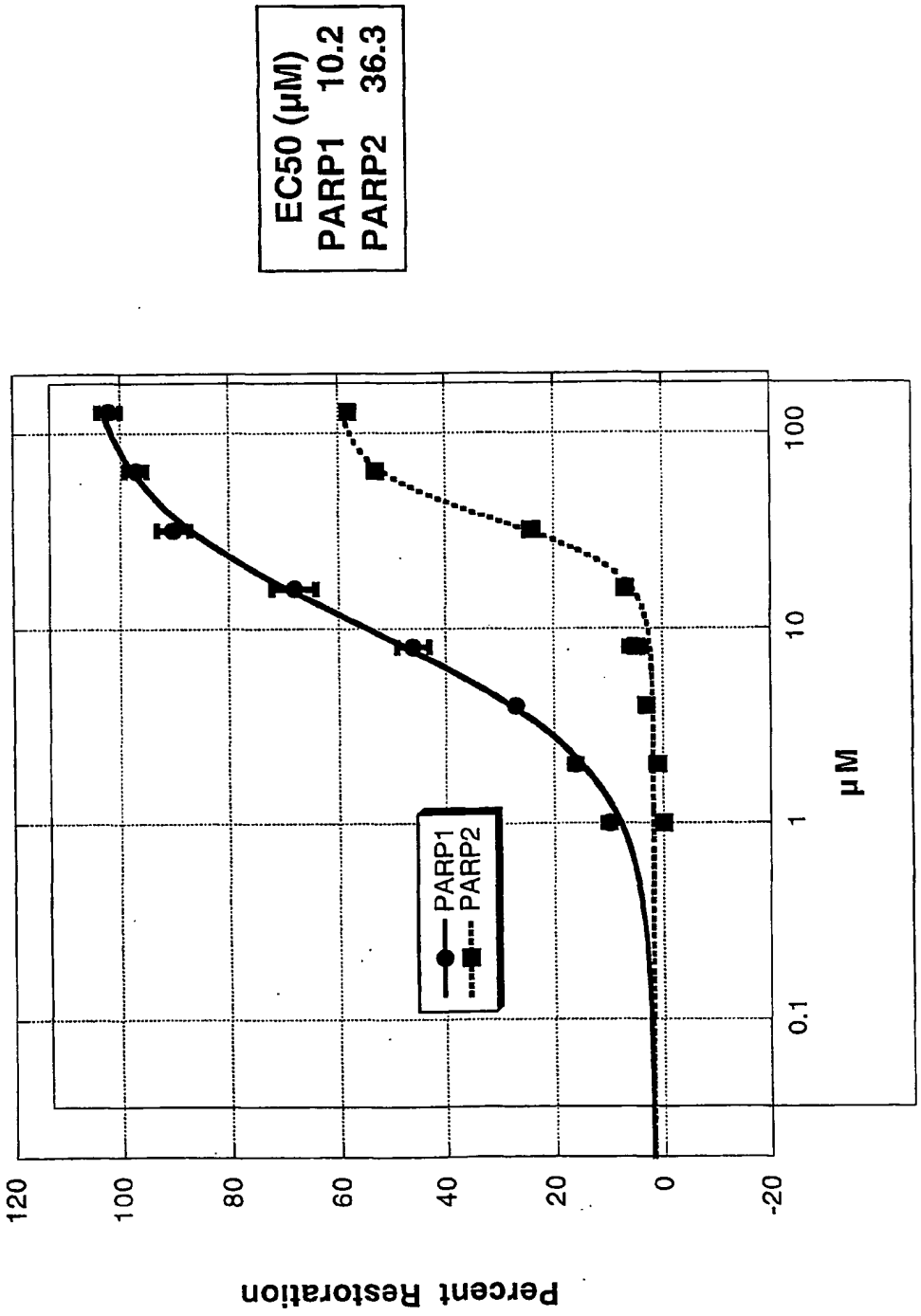

EC50 (μM)
PARP1 10.2
PARP2 36.3
Percent Restoration
120
100
80
60
40
20
0
-20
0.1
1
10
100
μM
PARP1
PARP2

Fig. 4

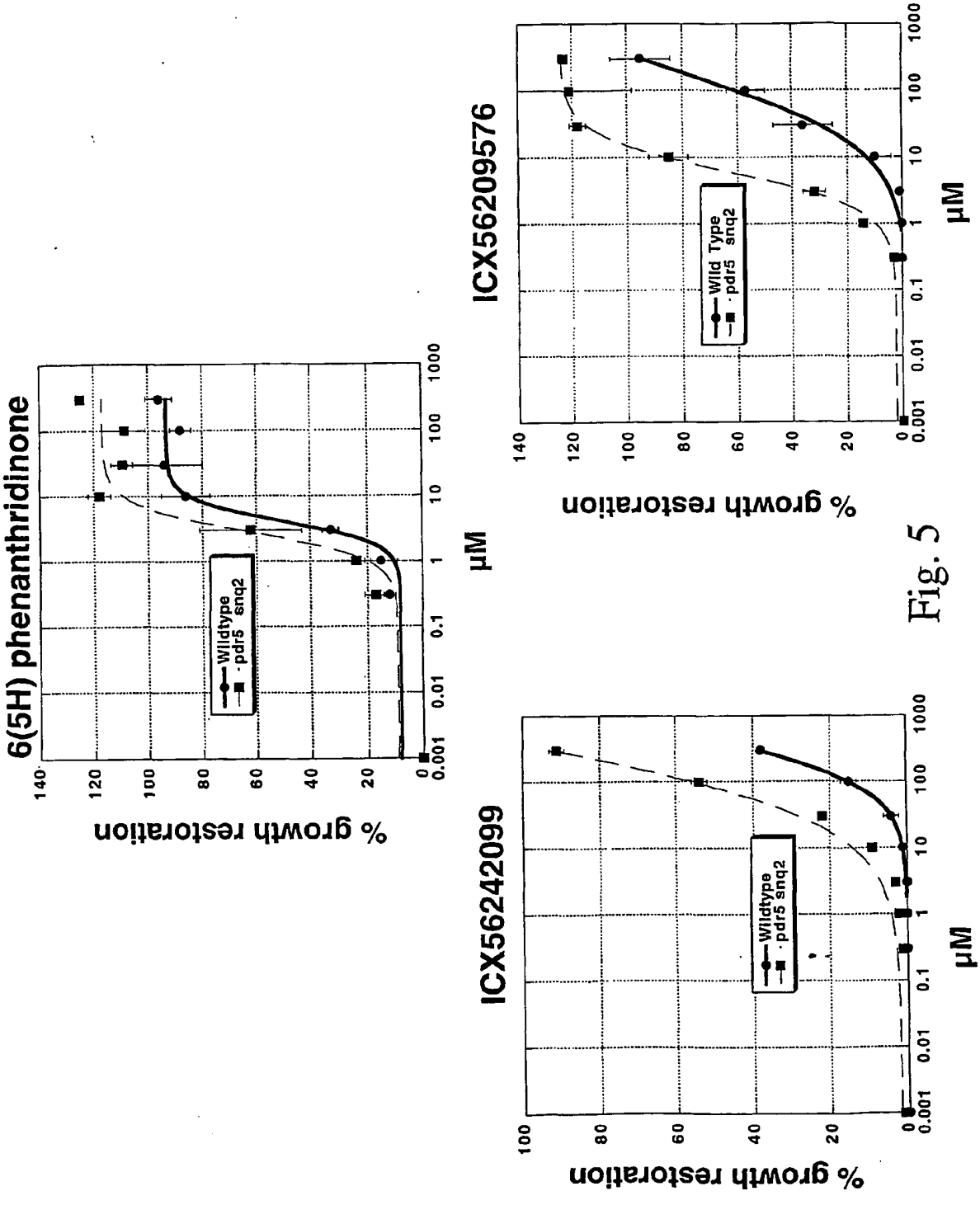

6(5H) phenanthridinone
% growth restoration
μM
Wildtype
pdr5 snq2
ICX56209576
% growth restoration
μM
Wild Type
pdr5 snq2
ICX56242099
% growth restoration
μM
Wildtype
pdr5 snq2

Fig. 5

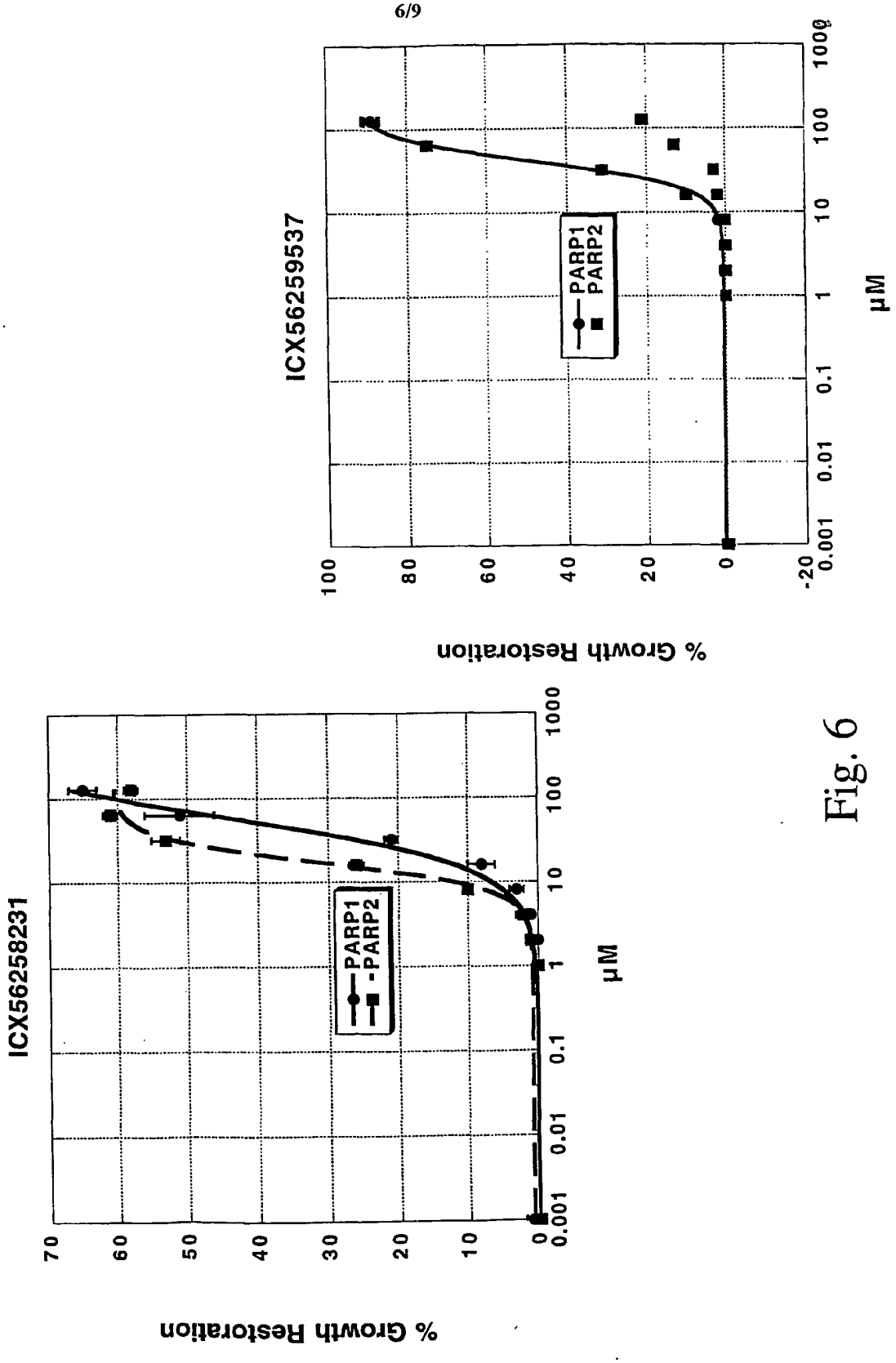

ICX56259537
PARP1
PARP2
% Growth Restoration
100
80
60
40
20
0
-20
0.001
0.01
0.1
1
10
100
1000
µM
ICX56258231
PARP1
PARP2
% Growth Restoration
70
60
50
40
30
20
10
0
0.001
0.01
0.1
1
10
100
1000
µM

Fig. 6

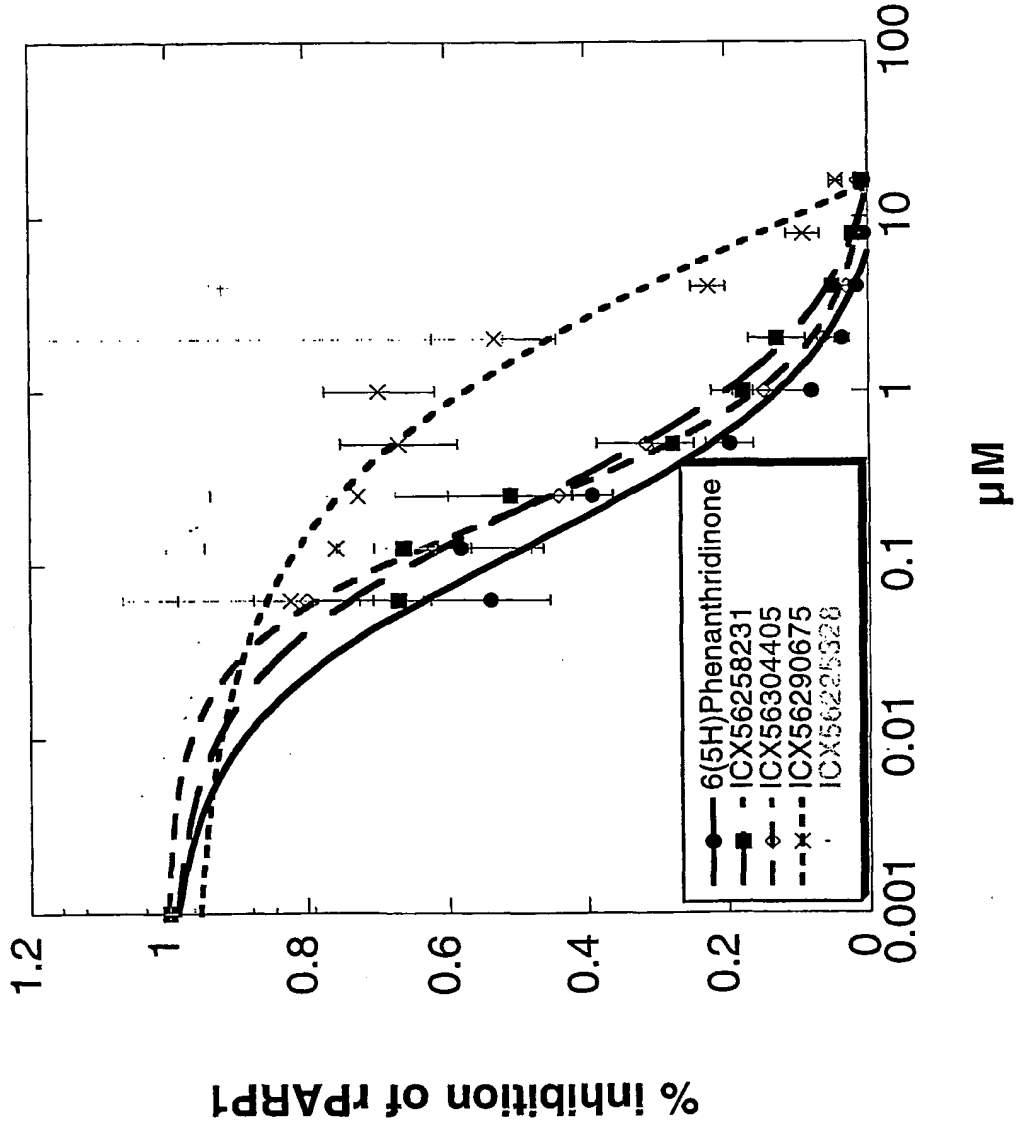

% inhibition of rPARP1
1.2
1
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
10
100
µM
6(5H)Phenanthridinone
ICX56258231
ICX56304405
ICX56290675

Fig. 7

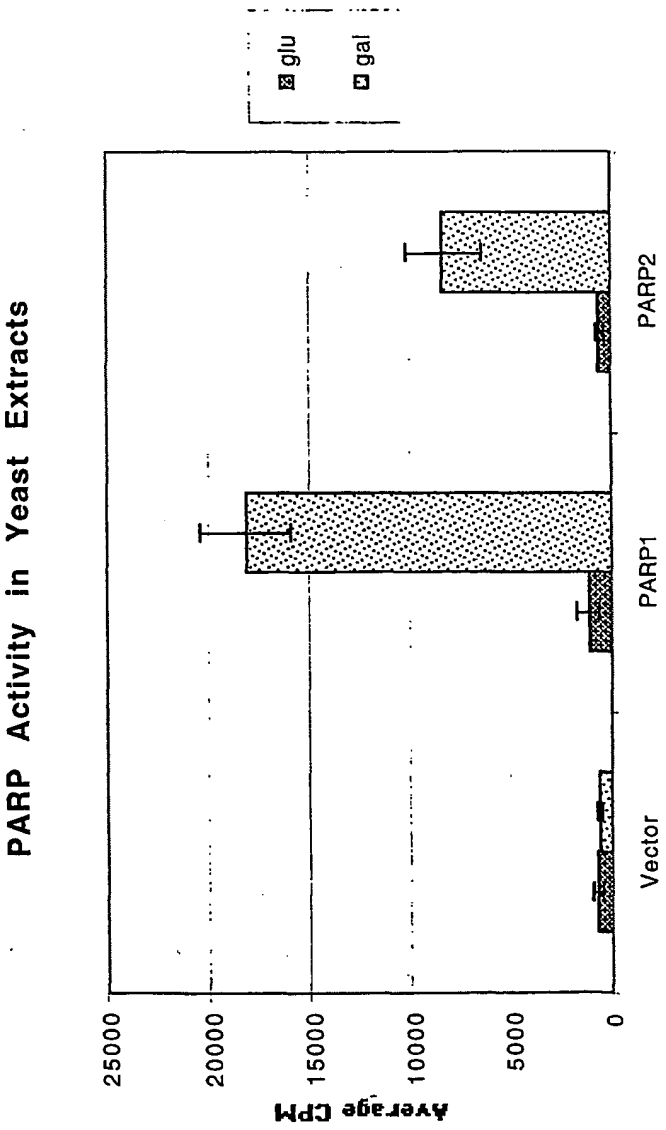

PARP Activity in Yeast Extracts
glu
gal
Average CPM
25000
20000
15000
10000
5000
0
Vector
PARP1
PARP2

Fig. 8

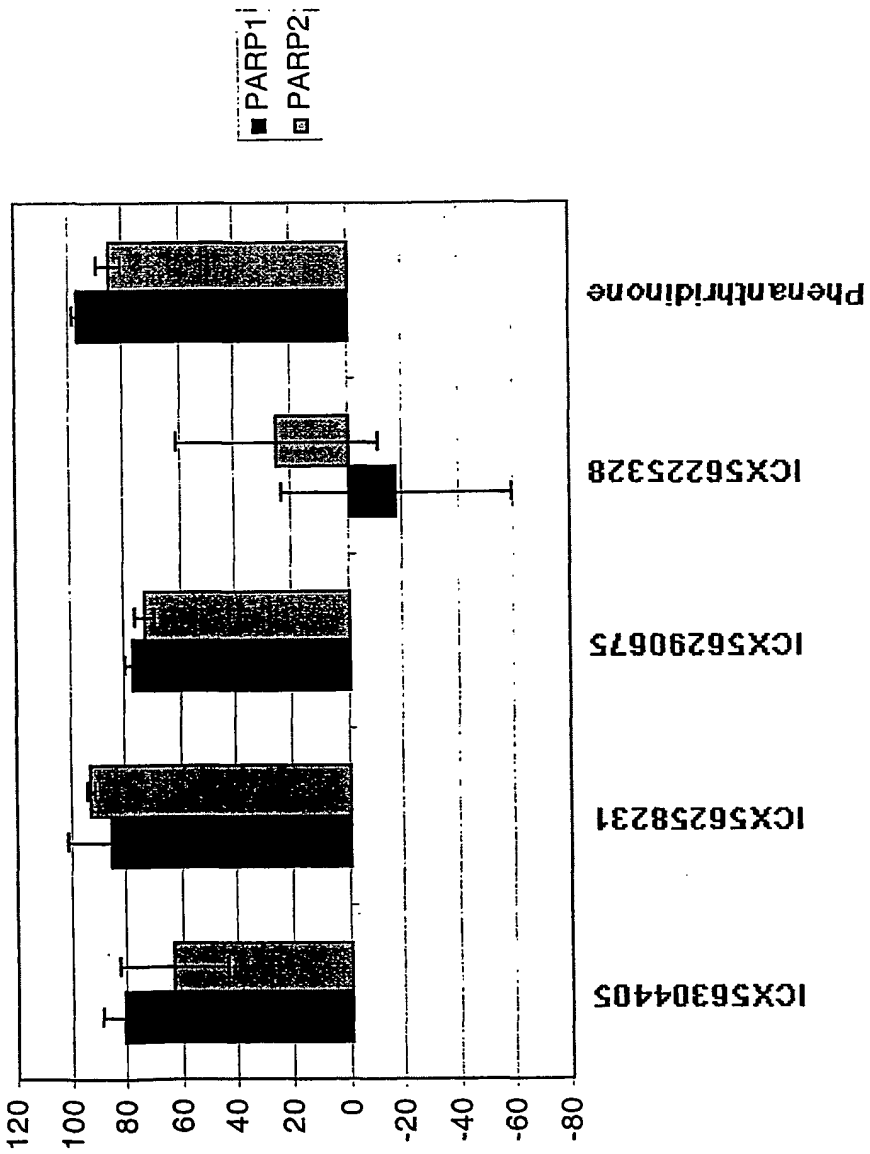

PARP1
PARP2
120
100
80
60
40
20
0
-20
-40
-60
-80
ICX563044O5
ICX562582 31
ICX562906 75
ICX562253 28
Phenanthridinone

Fig. 9

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International Bureau

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6 June 2002 (06.06.2002)

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(10) International Publication Number
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(21) International Application Number: PCT/US01/46811

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Filed on 1 December 2000 (01.12.2000)

(71) Applicant *(for all designated States except US)*: **ICONIX PHARMACEUTICALS, INC.** [US/US]; 850 Maude Avenue, Mountain View, CA 94043 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **MELESE, Teri** [US/US]; 850 Maude Avenue, Mountain View, CA 94043 (US). **PERKINS, Edward, L.** [US/US]; 850 Maude Avenue, Mountain View, CA 94043 (US). **YEH, Elaine** [US/US]; 850 Maude Avenue, Nountain View, CA 94043 (US). **SUN, Donxu** [CN/US]; 850 Maude Avenue, Mountain View, CA 94043 (US).

(74) Agents: **ROBINS, Roberta, L.** et al.; Robins & Pasternak LLP, 545 Middlefield Road, Suite 180, Menlo Park, CA 94025 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZM, ZW.

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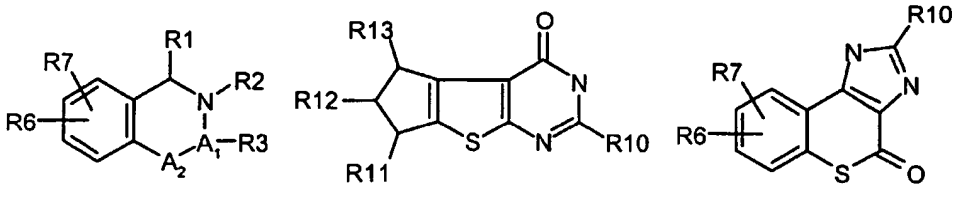

(57) Abstract: Compounds of formula 1, 2, and 3 where A_1 is $C(R_4)$or N; A_2 is $C(R_5)$ or S; R_1 is H, lower alkyl, halo, or a carbonyl; R_2 is H, lower alkyl, acyl, or forms a double bond with an adjacent ring atom; R_3 is H, lower alkyl, halo, aryl, aralkyl, acyl, lower alkenyl, or a radical or the form $(CH_2)nC(O)$-R_8, where R_8 is lower alkyl, OH, NH_2, lower alkoxy, lower aklylamino, di(lower alkyl)amino, aryl, or heterocyclyl, or forms a double bond with an adjacent ring atom; R_4 is H, lower alkyl, or forms a double bond with an adjacent ring atom, R_5 is H, lower alkyl, OH, halo, lower alkoxy, lower alkyl-thio, aryl-thio, or heterocyclyl-thio; R_6 and R_7 are each independently H, lower alkyl, OH, lower alkoxy, halo, nitro, amino, thio, acyl, lower alkylamino, acyloxy, acylamino, sulfinyl, sulfonyl, alkylsulfinyl, alkylsulfonyl, arylsulfonyl, aryl, heterocyclyl, aralkyl, or heterocyclyl-alkyl; R_{10} is H, lower alkyl, lower alkenyl, aryl, heterocyclyl, aryl-lower alkyl, or heterocyclyl-lower alkyl; and R_{11}, R_{12}, and R_{13} are each independently halo, nitro, OH, NH_2, or lower alkyl, and pharmaceutically acceptable salts thereof, are effective modulators of PARP enzymes.

WO 02/044157 A3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/46811

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07D237/32 C07D401/12 C07D409/04 C07D401/06 C07D217/24
C07D279/08 C07D239/90 A61K31/50 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07D A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 539 805 A (ASTA MEDICA) 5 May 1993 (1993-05-05) claims; table 1	1,2,11
X	WO 00 05218 A (ZAMBON GR.) 3 February 2000 (2000-02-03) page 1 -page 13; claims; examples 3,10,58	1,11,12
X	WO 99 11628 A (GUILFORD PHARMA.) 11 March 1999 (1999-03-11) page 25; claims 1,40-63	1,11,12
X	WO 98 33802 A (NEWCASTLE UNIV.) 6 August 1998 (1998-08-06) the whole document	1,8,11, 12

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
9 October 2002	25/10/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Francois, J

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 119, no. 3, 1993 Columbus, Ohio, US; abstract no. 20024q, HALL,I.: "THE ANTINEOPLASTIC ACTIVITY OF 2,3-DIHYDROPHTALAZINE-1,4-DIONE" page 32; column 2; XP002208916 abstract & ANTI-CANCER DRUGS, vol. 3, no. 1, 1992, pages 55-62, ENGL.	1,3,11, 12
X	GRIFFIN,R.: "RESISTANCE MODIFYING AGENTS.5." JOURNAL OF MEDICINAL CHEMISTRY., vol. 41, 1998, pages 5247-56, XP002208913 AMERICAN CHEMICAL SOCIETY. WASHINGTON., US ISSN: 0022-2623 page 5247 -page 5255	1,6,8, 11,12
X	GERHARD SATZINGER: "HETEROCYCLEN DURCH REAKTION VON MERCAPTO-U. HYDROXYCARBONSÄUREESTERN MIT AKT. NITRILEN." LIEBIGS ANNALEN DER CHEMIE., 1978, pages 473-511, XP002208914 VERLAG CHEMIE GMBH. WEINHEIM., DE ISSN: 0170-2041 page 501; figure FORMELSCH.1; example 26; table 11	1,7
X	GRIFFIN, R.: "NOVEL BENZIMIDAZOLE A. QUINAZOLINONE INHIBITORS" PHARMACEUTICAL SCIENCES, vol. 2, no. 1, 10 January 1996 (1996-01-10), pages 43-7, XP000886156 page 43 -page 46	1,8,11, 12
X	HALL, I.: "THE CYCLOTOXIC ACTIVITY OF CYCLIC IMIDO ALKYL ETHERS" ANTI-CANCER DRUGS, vol. 5, no. 1, February 1994 (1994-02), pages 75-82, XP008006442 ENGL. page 75 -page 81	1-3,6, 11,12

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4

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IBRAHIM, NADIA S.: "NITRILES IN HETEROCYCLIC SYNTHESIS" HETEROCYCLES., vol. 22, no. 8, 1984, pages 1677-82, XP008006446 ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM., NL ISSN: 0385-5414 page 1677 -page 1680 ---	1,7
P,X	ED. PERKINS ET AL.: "NOVEL INHIBITORS OF POLY(ADP-RIBOSE) POLYMERASE/PARP1 A. PARP2" CANCER RESEARCH, vol. 61, no. 10, 15 May 2001 (2001-05-15), pages 4175-83, XP002208915 the whole document ---	1-12
E	WO 02 09681 A (MEDINKOR ZMM) 7 February 2002 (2002-02-07) claims -----	1,3,11, 12

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 01/46811

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

In view of the large number and also the wording of the claims presently on file, which render it difficult, if not impossible, to determine the matter for which protection is sought, the present application fails to comply with the clarity and conciseness requirements of Article 6 PCT (see also Rule 6.1(a) PCT) to such an extent that a meaningful search is impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear (and concise), namely the compounds of the examples and related homologues.We excluded the compounds where R1 to R7 are all H together for instance.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

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International Application No
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